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(54) Title: METHOD FOR CONTROLLING MOLECULAR WEIGHT OF POLYHYDROXYALKANOATES

(57) Abstract

A method has been developed for control of molecular weight and molecular weight dispersity during production of polyhydroxyalkanoates in genetically engineered organism by control of the level and time of expression of one or more PHA synthases in the organisms. The method was demonstrated by constructing a synthetic operon for PHA production in *E. coli* in which the level of PHA synthase activity could be tightly controlled by placement of the synthase behind an inducible promoter. Modulation of the total level of PHA synthase activity in the host cell by varying the concentration of the inducer, isopropyl β-D-thiogalactoside (IPTG), was found to effect the molecular weight of the polymer produced in the cell. Specifically, high concentrations of synthase activity were found to yield polymers of low molecular weight while low concentrations of synthase activity yielded polymers of higher molecular weight. Polymer molecular weight dispersity is also proportional to the amount of synthase activity, with less dispersity in polyhydroxyalkanoate compositions produced in expression systems with an initial burst of synthase activity, and higher levels of molecular weight dispersity in polyhydroxyalkanoate compositions produced in expression systems with the levels of synthase activity varied during synthesis of the polyhydroxyalkanoate.

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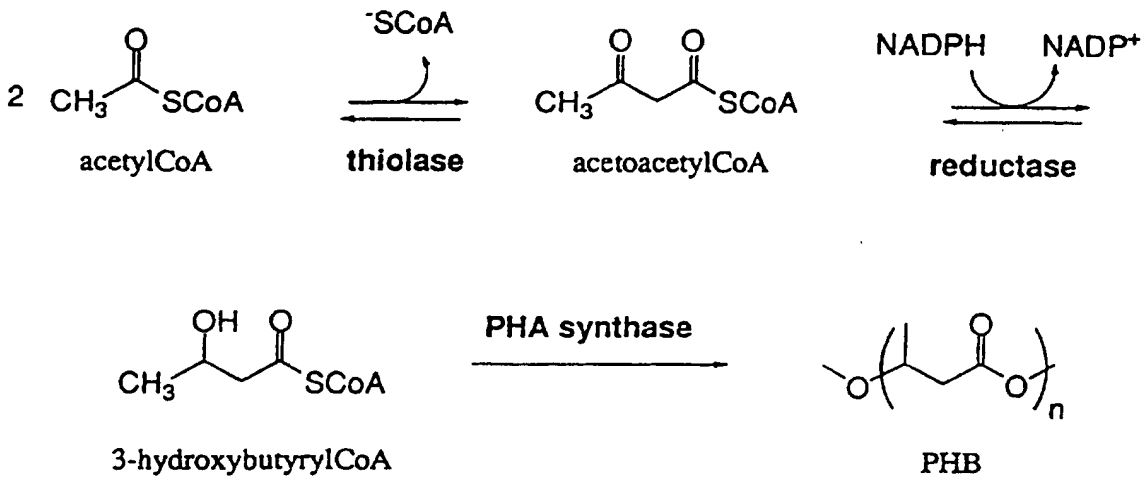
METHOD FOR CONTROLLING MOLECULAR WEIGHT OF POLYHYDROXYALKANOATES

Background of the Invention

This invention relates to controlling the molecular weight and distribution of polyhydroxyalkanoates produced *in vivo* in a host cell by genetically engineering the amount of polyhydroxyalkanoate polymerase which is expressed in the host cell.

The United States government has certain rights in this invention by virtue of National Institutes of Health grant NIH-1-RO1-GM49171-01A1 to JoAnne Stubbe, A.J. Sinskey, and S. Masamune

Polyhydroxyalkanoates (PHAs) are a class of polyesters that are produced by many organisms as an intracellular energy reserve during nutrient deprivation (Anderson and Dawes, Microbiological Reviews, 1990, 54, 450-472.; Poirier et al., Bio/Technology 1995, 13, 142-150.). Since PHAs are naturally biodegradable, they are considered an attractive alternative to conventional plastics in terms of reducing solid waste. *Alcaligenes eutrophus* is an example of an organism that produces abundant granules of the polyester during nutrient limiting conditions. The PHA biosynthetic pathway in *A. eutrophus* consists of three enzymes, a thiolase, a reductase, and a synthase. Two acetyl-CoA units are condensed by β-ketothiolase to form acetoacetyl-CoA, which is stereospecifically reduced by an NADPH dependent reductase to form 3-D-hydroxybutyryl-CoA (HBCoA). PHA synthase catalyzes the polymerization of HBCoA monomer units to form polymer.



The operon containing the PHA biosynthetic genes from *A. eutrophus* has been cloned, allowing its expression in *Escherichia coli* (Peoples and Sinskey, J. Biol. Chem. 1989, 264, 15298-15303; J. Biol. Chem. 1989, 264, 15293-15297; Slater et al., J. Bacteriol. 1988, 170, 4431-4436; Schubert et al., J. Bacteriol. 1988, 170, 5837-5847). Wild-type *E. coli* is itself capable of producing small amounts of a form of polyhydroxybutyrate (PHB) that is found complexed to the cell membranes (Huang and Reusch, J. Bacteriol. 1993, 177, 486-490), however the abundant granules found in other microorganisms such as *A. eutrophus* during nutrient limiting conditions are not present in *E. coli*. Insertion of a plasmid-borne copy of the PHA biosynthetic operon from *A. eutrophus* enables *E. coli* to produce PHB granules visible by transmission electron microscopy (Lee et al., 1994a). Considerable attention has been devoted to the fermentation of genetically engineered *E. coli* PHB producers (Lee et al., 1994a and Lee, et al., Journal of Biotechnology 1994, 32, 203-211; Kim et al., Biotechnology Letters, 1992, 14, 811-818.; Kidwell et al., Applied and Environmental Microbiology 1995, 61, 1391-1398), however little effort has been made to characterize the size of the polymer produced or to develop methods to control polymer molecular weight. As reviewed by Gilbert, "Molecular Weight Distributions in Free-Radical Polymerizations: Their Cause and

Cure" Trends in Polymer Science 1995, 3, 222-226, the average molecular weight and the molecular weight distribution are some of the most important determinants of the properties of a polymer.

Gerngross and Martin, Proc. Natl. Acad. Sci. USA 92:6279-6283 (July 1995), describe a method for increasing the molecular mass of poly[(R)-(-)-3-hydroxybutyrate] (PHB) granules by exposing purified PHA synthase to (R)-3-hydroxybutyryl coenzyme A, and determined that the molecular mass of the polymer is dependent on the initial PHA synthase concentration in a cell free, *in vitro* system wherein specific substrate is added. While this is an interesting research observation, it provides little basis on which commercial synthesis of polymer of defined molecular weight can be produced in a bacterial system where many other enzymes and substrates are present. The substrate needed for *in vitro* polymerization, D-3-hydroxybutyrylCoA, is only commercially available as a mixture of D and L diasteriosomers. The current cost of D,L-3-hydroxybutyrylCoA from Sigma Chemical Company, St. Louis, MO, is $187.55 for 25 milligrams of material.

Huisman, in his Ph.D. thesis entitled "Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant strains of *Pseudomonas putida*" submitted to the University of Groningen, The Netherlands, 1991, and Huisman, et al., Appl. Microbiol. Biotechnol. 38:1-5 (1992), analyzed the poly(3-hydroxyalkanoates) produced in mutants defective in PHA metabolism. Comparisons of molecular weights and quantities of polymer were made based on the substrate provided to the mutant. It was postulated that, in addition to substrate, the polymerase played a role in determining the molecular weight of the resulting polymer. Average PHA molecular weight was theorized to be inversely proportional to polymerase activity, but the exact role and its relationship to substrate availability in final product characteristics were not determined. Moreover, the studies were conducted using plasmid-based expression of PHA synthase genes from one strain, *Pseudomonas oleovarans*, in a host of a different strain, *P. putida*, a system that does not necessarily guarantee expression. No

enzyme activity data was provided to support claims of expression in this heterologous system. The strain is also capable of degrading polymer. Indeed, the results suggest degradation by depolymerases, not control of synthase activity, since polymer yields decrease with decreases in molecular weight.

Although a correlation between molecular weight of the resulting polymer and concentration of PHA synthase has been suggested, many factors, including substrate availability, clearly play a role.

It is therefore an object of this invention to provide a method for controlling polymer molecular weight and molecular weight dispersity.

Summary of the Invention

A method has been developed for control of molecular weight and molecular weight dispersity during production of polyhydroxyalkanoates in genetically engineered organisms by control of the level and time of expression of one or more PHA synthases in the organisms. The method was demonstrated by constructing a synthetic operon for PHA production in *E. coli* in which the level of PHA synthase activity could be tightly controlled by placement of the synthase behind an inducible promoter. Modulation of the total level of PHA synthase activity in the host cell by varying the concentration of the inducer, isopropyl β-D-thiogalactoside (IPTG), was found to effect the molecular weight of the polymer produced in the cell. Specifically, high concentrations of synthase activity were found to yield polymers of low molecular weight while low concentrations of synthase activity yielded polymers of higher molecular weight. Polymer molecular weight dispersity can be controlled by modulating the synthase activity during the polymer synthesis reaction. For example, low synthase activity at the beginning of the fermentation yields high molecular weight polymer of low molecular weight dispersity; increasing synthase activity during fermentation results in synthesis of lower molecular weight polymer, thereby increasing the molecular weight dispersity of the polymer produced during the fermentation. Examples

demonstrate the synthesis of polymers having a range of molecular weights between 4 x 10^6 and 7.8 x 10^5, and defined molecular weight distributions (polydispersity indices from 1.02 and 2.81).

Brief Description of the Drawings

Figures 1A, 1B, and 1C are graphs of dry cell weight (DCW), PHB concentration (PHB), and glucose concentration during 13 L fermentations of strains DH5α/pAeT41 (Figure 1A), DH5α/pSP2 with 0.4 mM IPTG induction (Figure 1B), and DH5α/pSP2 with 5 mM IPTG induction (Figure 1C).

Figure 2 is a graph of enzyme activities (specific activity, units/mg, time, hour) of the synthase in soluble lysate fractions of strains DH5α/pAeT41 (triangles), DH5α/pSP2 with 0.4 mM IPTG induction (circle), and DH5α/pSP2 with 5 mM IPTG induction (solid circle).

Figure 3A is a graph of synthase activity (specific activity, units/mg over time, hour) for crude lysates of strains DH5α/pAeT41 (triangle), DH5α/pSP2 with 0.4 mM IPTG induction (circle), and DH5α/pSP2 with 5 mM IPTG induction (solid circle). The crude lysate is defined as the lysate fraction containing both soluble and insoluble enzyme fractions.

Figure 3B is a graph illustrating multi-angle light scattering molecular weight analysis of polymer isolated from strains DH5α/pAeT41 (triangle), DH5α/pSP2 with 0.4 mM IPTG induction (circles), and DH5α/pSP2 with 5 mM IPTG induction (solid circles).

Figure 4 is a graph of the activity of the reductase (specific activity, units/mg, over time, hours) in soluble lysate fractions of strains DH5α/pAeT41 (triangle), DH5α/pSP2 with 0.4 mM IPTG induction (circle), and DH5α/pSP2 with 5 mM IPTG induction (solid circle).

Figure 5 is a graph of the activity of the thiolase in soluble lysate fractions of strains DH5α/pAeT41 (triangle), DH5α/pSP2 with 0.4 mM IPTG induction (circles), and DH5α/pSP2 with 5 mM IPTG induction (solid circles).

Figure 6A is a graph of the polydispersity index (Mw/Mn) over time (hours) of polymer isolated from strains DH5α/pAeT41 (triangles), DH5α/pSP2 with 0.4 mM IPTG induction (circles), and DH5α/pSP2 with 5mM IPTG induction (solid circles), as calculated by gel permeation chromatography in line with light scattering. Figures 6B and 6C are raw gel permeation chromatograms performed without light scattering of polymer isolated from strain DH5α/pSP2 induced with 0.4 mM IPTG (Figure 6B) and polymer isolated from strain DH5α/pSP2 induced with 5 mM IPTG (Figure 6C).

Figures 7A and 7B are schematics of the construction of plasmid pSP2.

Detailed Description of the Invention

The polyhydroxyalkanoates (PHAs) detected in bacteria can be divided into two main groups, those containing short chain monomer units (3-5 carbon atoms) and those containing medium chain monomer units (6-14 carbon atoms) (Lee, S. Y. Biotechnology and Bioengineering 49, 1-14, 1996). The synthase enzymes are generally divided into three classes: Type I, such as *A. eutrophus*, that synthesize polymer with short chain monomer units; Type II, such as *Pseudomonas oleovorans*, that synthesize polymer containing medium chain monomer units; and Type III, such as *Chromatium vinosum*, that require the expression of a second gene *phaE* for synthase activity and predominantly synthesize short chain polymers. There has been a synthase found in the type III group from *T. pfennigii* that synthesizes a copolymer composed of short and medium chain length monomer units (Poirier et al., Bio/Technology 13, 142-150, 1996).

Short chain monomer units that are recognized as substrates by the short chain PHA synthases include 3, 4, and 5-hydroxyacyl CoAs [Lee, S. Y. Biotechnology and Bioengineering 49, 1-14, 1996]. In general, the presence of a thiolase and reductase is needed to make polymer in short chain production systems. For example, 3-hydroxybutrylCoA is formed from acetylCoA via 3-ketothiolase and acetoacetyl reductase. Other short

chain monomer units can be formed by adding alkanoic acids or hydroxyalkanoic acids to the bacterial growth medium. Alkanoic acids, such as propionic acid, are converted to acyl CoAs, via a CoA synthase, which are subsequently converted to hydroxyacylCoA's via a thiolase and reductase (Poirier et al., Bio/Technology 13, 142-150, 1995). However, hydroxyalkanoic acids are converted to hydroxyacylCoAs by the bacteria without the need of thiolase or reductase enzymes.

Monomer units of medium chain length PHAs are derived either from alkanoic acids added to the bacterial growth medium or from the fatty acid biosynthetic and β-oxidation pathways when the bacteria are grown on simple sugars. *P. oleovorans* is an exception in that it has not been found to produce PHAs when grown on simple sugars (Poirier et al., Bio/Technology 13, 142-150, 1995). Acyl CoAs, formed from the alkanoic acid via an acyl CoA synthase, are converted to hydroxyacyl CoAs by the β-oxidation pathway (Poirier et al., Bio/Technology 13, 142-50, 1995). Thus, medium chain length polymer production systems do not need the thiolase and reductase, however, they do require the presence of β-oxidation and fatty acid biosynthetic pathway enzymes.

Molecular weight control by controlling synthase activity applies to all classes of polymers, not just PHB, as well as all classes of synthases.

PHA Synthases

Many different PHA synthases are known, characterized and the encoding DNA is sequenced. These are generally derived from natural sources, but can include synthases with conservative substitutions in the amino acid sequences.

References for sequences of known synthases include: *Paracoccus denitrificans*: Ueda, et al., Journal of Bacteriology 1996, 178, 774-779; *A. eutrophus*: Peoples, et al., J. Biol. Chem. 1989, 264, 15293-15297 (Genbank accession number JO5003); *Acinetobacter* sp.: Schembri, et al., FEMS Microbiol. Lett. 1994, 118, 145-152; *Chromatium vinosum*: Liebergesell, et al., Eur. J. Biochem. 1992, 209, 135-150; *M. extorquens*: Valentin, et al., Appl. Microbiol. Biotechnol.

1993, 39, 309-317; *Pseudomonas aeruginosa* 1 and 2: Timm, et al. Eur. J. Biochem, 1992, 209, 15-30; *Pseudomonas oleovorans* 1 and 2: Huisman, J. Biol. Chem. 1991, 266, 2191-2198; *Rhodococcus ruber*: Pieper, et al., FEMS Microbiol. Lett., 1992, 96, 73-80; *R. sphaeroides*: Hustede, et al., Biotechnol. Lett. 1993, 15, 709-714; *Thiocystis violacea*: Liebergesell, et al., 1993, 38, 493-501.

Thiolases and Reductases

A. eutrophus thiolase and reductase are described by Peoples and Sinskey, J. Biol. Chem., 264, 15293-15297 (1989) and available from Genbank under the accession number JO4987.

Depolymerases

In *E. coli* it is not necessary to inactivate the depolymerase since there is no known depolymerase and degradation has not been observed. However, in other host strains where a functional depolymerase exists, it would be desirable to eliminate the depolymerase so that the polymer product does not degrade. A depolymerase can be inactivated by mutagenesis of the host strain.

Expression Vectors

The gene encoding the synthase, as well as any other enzymes such as the reductase and thiolase, can be inserted into an appropriate vector for expression in a host system. In the preferred embodiment, the vector is a plasmid expressable in a bacteria such as *Escherichia coli*. Suitable plasmids can be obtained from commercial sources including the American Type Culture Collection, Rockville, MD. Other commercial sources for expression vectors include Pharmacia Biotech Inc., Piscataway, NJ; Clontech Laboratories, Inc., Palo Alto, California; Promega, Madison, WI.

In the preferred embodiment, expression of the synthase is under the control of an inducible promoter, as demonstrated by the following examples. Promoters that can be utilized to regulate the expression of PHA synthase in bacterial hosts include, but are not limited to, an IPTG inducible promoter such as the *lac, trc, or tac* promoters; a pH regulated

promoter regulated by pH (Tolentino et al, Biotechnology Letters, 14, 157-162, 1992); a pH regulated promoter regulated by aerobic or anaerobic conditions (Tolentino et al.); a pH regulated promoter regulated by combining pH control with anaerobic/aerobic conditions (Tolentino et al.); an arabinose controlled promoter regulated by arabinose levels in culture media (Guzman et al., J. Bacteriol. 177, 4121-4130, 1995); repression of an arabinose controlled promoter with glucose yielding extremely low levels of synthase expression Guzman et al.); a T7 RNA polymerase promoter such as ϕ10 regulated by T7 RNA polymerase; the promoter regions of heat shock genes such as *dnaK, grpE,* and *lon*, that can be induced either through heat shock or other environmental stresses such as the addition of chemical inducers such as ethanol (Van Dyk et al., J. Bacteriol. 177, 6001-6004, 1995).

Expression Systems

In the preferred embodiment, the host is a bacteria which has been genetically engineered so that it secretes polymer rather than accumulating it in insoluble granules, for example, by genetically engineering the host cell with a gene that allows lysis of the cells when induced. The most preferred host is *E. coli*. Others include Gram positive bacteria such as *Corynebacterium*, *Pseudomonas*, eukaryotic cells, and plant hosts.

Prokaryotic, eukaryotic and plant systems can be created to produce a range of polymers possessing different molecular weights and polydispersities by placing promoters in front of the synthase gene that are inducible to different levels. Alternatively, a series of separate strains, cell lines, or plants containing constitutive promoters of different strengths could be created such that each strain, cell line, or plant produces one polymer product of the desired molecular weight and polydispersity.

In bacterial hosts, synthase activity can be further regulated by employing constituative or inducible promoters in front of synthase genes in low copy number plasmids, such as those containing the pSC101 replicon, medium copy number plasmids, such as those containing the

pMB1, p15A, or ColEl replicons, high copy number plasmids, such as the pUC plasmids containing a mutated pMBl replicon, chromosomal insertion of a single copy of the gene into a host strain, or chromosomal insertion of multiple copies of the gene into a host strain.

In plants, expression systems for generating polymers possessing different molecular weights can include the construction of a stably transformed plant containing genes encoding the reductase and thiolase, in which the synthase gene is located behind an inducible promoter, such as the soybean heat-shock promoter Gmgsp17.3. Alternatively, a series of individual stably transformed plants can be created containing genes encoding the reductase and thiolase and in which the PHA synthase gene(s) are behind constituative promoters of varying strength such that each plant has a different synthase activity and therefore produces a polymer of different molecular weight. PHA production can be targeted to the plastids of the plants by fusing a transit peptide, such as the pea chloroplast transit peptide, to the thiolase, reductase, and synthase enzymes, a strategy that has been shown to increase polymer yields in transgenic plants (Nawrath et al., Proc. Natl. Acad. Sci USA 91, 12760-12764, 1994).

Fermentation Conditions

Fermentation conditions that can be used for producing PHA's in bacteria include batch fermentation and continuous culture. Carbon sources for either fermentation can be simple sugars or alkanoic or hydroxyalkanoic acids supplemented to the culture medium depending on the type of PHA that is desired and the genetic background of the host production organism. For example, the co-polymer of 3-hydroxybutyrate and 3-hydroxyvalerate can be formed in *E. coli* by adding propionic acid to the culture medium of an *atoC fadR* mutant strain (Rhie and Dennis, Applied and Environmental Microbiology, 1995, 61, 2487-2492).

The data presented in the examples led to the identification of PHA synthase as the component responsible for controlling the molecular weight of PHA biosynthesized in genetically recombinant bacteria such as

E. coli. Two constructs were examined, DH5α/pAeT41, containing the native *A. eutrophus* PHA biosynthetic operon, and DH5α/pSP2, containing a modified operon in which the ribosome binding site in front of the synthase has been optimized and the transcription of the PHA biosynthetic genes is under the control of an inducible promoter. The role of the synthase in determining the molecular weight of the polymer is most evident upon comparison of the fermentations of DH5α/pAeT41 and DH5α/pSP2, induced with 0.4 mM IPTG. Synthase activity, both soluble and insoluble, remains at a constant undetectable level throughout the fermentation of DH5α/pAeT41, yielding high molecular weight polymer of approximately constant size at the beginning (4.3×10^6 Da) and end (4.0×10^6 Da) of the fermentation. In contrast, an increase in insoluble synthase activity in DH5α/pSP2 (0.4 mM IPTG) is observed coincident with a decrease in polymer size, yielding polymer of significantly smaller size at the end of the fermentation (1.3×10^6 Da) than at the beginning (2.2×10^6 Da). Approximately equal levels of thiolase activity and little or no reductase activity are observed in both fermentations indicating that these enzymes are not responsible for the observed difference in molecular weight. The role of the synthase is further substantiated in the fermentation of DH5α/pSP2 induced with 5 mM IPTG in which *in vivo* synthase activities are further increased, yielding polymer of still lower molecular weight (7.3×10^5 Da) at the end of the fermentation.

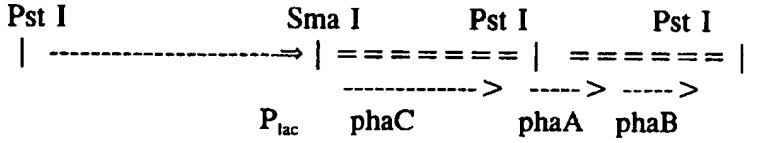
Although the described methodology for controlling molecular weight *in vivo* is illustrated with a genetically engineered strain of *E. coli* and PHB, it is not limited to *E. coli* or PHB and can be used in any organism expressing either native or genetically engineered PHA biosynthetic genes to produce any member of the class of polymers encompassed by polyhydroxyalkanoates. The control of PHA synthase activity is not limited to induction of an inducible promoter with IPTG, for any method capable of altering the *in vivo* concentration of PHA synthase activity is capable of controlling the molecular weight of polymer produced in the respective organism.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Production and Characterization of PHB Produced in *E. coli*.

Methods and Materials:

Fermentation of *E. coli* DH5α/pAeT41 was used to establish a reference from which to compare all other genetically engineered *E. coli* PHB producers. Plasmid pAeT41, described in U.S. Patent No. 5,245,023 to Peoples, et al., contains a 5 kb genetic fragment encoding the native, unmodified PHA biosynthetic operon from *A. eutrophus* inserted into pUC18, Sequence Listing ID No. 1. pAeT41 (7.7 kb) is shown schematically as:



As with all other plasmids described in this study, plasmid pAeT41 was transformed into the *E. coli* strain DH5α for polyester production. Strains were cultured at 30°C in a 15 L MCS-10 Bioreactor AG fermentor (MBR, Switzerland) in LB media (LB media contains per L, 10 g tryptone, 5 g yeast extract, and 10 g NaCl) supplemented with glucose (20 g/L) and ampicillin (50 mg/mL), As with all other fermentations in this study, aliquots of cells were removed for enzyme activity analysis at time points throughout the fermentation, pelleted by centrifugation, and frozen at -70°C. Samples were thawed, resuspended in M9 salts (M9 salts contain, per L, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl), and divided into three individual samples for the assay of synthase, thiolase, and reductase activity. Cells were isolated by centrifugation, resuspended in lysis buffer, and disrupted by sonication using an Ultrasonic Processor xL (Farmingdale, NY). After lysis, samples were centrifuged at 4°C for 15 min to isolate soluble protein fractions.

PHA synthase was assayed by monitoring coenzymeA release using 5, 5'-dithiobis(2-nitrobenzoic acid) as described by Gerngross et al. Biochemistry, 1994, 33, 9311-9320. Both the soluble protein fraction and the crude sonicated lysate were assayed, allowing the determination of both soluble and insoluble synthase enzyme activities. Prior to sonication, samples for the assay of PHA synthase were resuspended in 50 mM KH_2PO_4 (pH 7.0) containing 5% glycerol. AcetoacetylCoA reductase was assayed by monitoring the oxidation of NADPH as described by Ploux, et al., Eur. J. Biochem. 1988, 174, 177-182, with the exception that the acetoacetylCoA concentration was increased to 1.8 mM. Prior to sonication, samples for the assay of reductase activity were resuspended in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 10 mM β-mercaptoethanol, 20% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride. β-ketothiolase activity was assayed by measuring the rate of NAD^+ formation as described by Nishimura et al., Arch. Microbiol. 1978, 116, 21-27. Prior to sonication, samples for the assay of thiolase activity were resuspended in 10 mM Tris-Cl (pH 7.3) containing 5 mM β-mercaptoethanol. The PHB content in fermentation broths was assayed as described by Law and Slepecky, J. Bacteriol. 1961, 82, 33-36. The glucose concentration in fermentation broths was determined using a 1050 Hewlett Packard HPLC system equipped with a model 1047A refractive index detector and a Bio-Rad Aminex HPX-87H column maintained at 40°C. Glucose was eluted with a mobile phase of 0.008N sulfuric acid at a flow rate of 0.6 mL/min.

During batch fermentation of DH5α/pAeT41 (Figure 1A), the initial 20 g/L of glucose supplied to the cells was completely consumed after 18.7 h. Dry cell weight and PHB concentration reached a maximum of 8.7 and 6.0 g/L after 18.7 and 23.3 h of fermentation, respectively. Despite localization of the *A. eutrophus* PHA biosynthetic operon on the high copy plasmid pUC18, no soluble PHA synthase activity was detected during the entire DH5α/pAeT41 fermentation (Figure 2). The presence of polymer in cell lysates often results in localization of synthase activity

in the insoluble portion of the lysate (Fukui et al., Microbiol. 1976, 110, 149-156.; Haywood et al., FEMS Microbiology Letters, 1989, 57, 1-6). An additional assay was thus performed for all fermentation samples by measuring the combined soluble and insoluble fractions in the crude lysate prior to centrifugation. DH5α/pAeT41 lysates assayed in this way still contained no detectable synthase activity (Figure 3A). Maximum reductase activity of 0.033 U/mg was observed at 18.7 h (Figure 4) while assays for thiolase activity yielded a maximum of 2.5 U/mg at the 14.6 h (Figure 5).

Polymer purification from samples for molecular weight analysis proceeded as follows. Cells were pelleted by centrifugation and washed with distilled water. Cells were collected by centrifugation, lyophilized, and refluxed in chloroform for 5 h. The cellular debris was removed from the polymer solution by filtration through a sintered glass funnel. PHB was precipitated from the chloroform solution with one volume of hexane, isolated by filtration through a sintered glass funnel, and dried overnight *in vacuo*. ^{1}H NMR analysis of the polymer dissolved in $CDCl_3$ confirmed that the isolated material was PHB in high purity [^{1}H NMR ($CDCl_3$; 300 MHz) δ=1.24(d, ξ=6.3Hz, 3H), 244 (dd, ξ=6.5, 15.5 Hz, 1H), 2.58 (dd, ξ-7.4, 15.5, 1 H), 5.23 (m, 1H)].

Gel permeation chromatography without light scattering (Figures 6B and 6C) was performed on a Progel-TSK G7000H_{XL} column in series with a Shodex K-805 GPC column. Polymer samples were eluted in chloroform at 1 mL/min at 30°C.

The weight average molecular weight (M_w) and Polydispersity index (PDI), equivalent to M_w/M_n where M_n is the number average molecular weight, were measured for each polymer sample by multi-angle light scattering. Light scattering was performed using a Waters model 501 pump and model 410 differential refractometer equipped with a Shodex K-807LC GPC column and a Dawn-F multi-angle laser photometer (Wyatt Technology). Polymer samples were eluted with 2,2,2-trifluoroethanol at 35°C. Polymethylmethacrylate standards were

used to normalize the instrument and light scattering analysis of the standards yielded molecular weight values in good agreement with those reported by the supplier.

Polymer samples isolated at various time points from the fermentation of DH5α/pAeT41 possessed M_w ranging from 4.3 X 10^6 to 4.0 X 10^6 Da with the exception of a small drop to 3.1 X 10^6 Da at the 23 h time point (Figure 3B), M_w values that are significantly larger than the 1.0 X 10^6 to 1.2 X 10^6 Da M_w observed for polyester isolated from routine fermentations of *A. eutrophus* (Kawaguchi and Doi, Macromolecules 1992, 25, 2324-2329.; Hahn et al., Biotechnology and Bioengineering, 1994, 44, 256-261). The polydispersity index ranged from 1.01 to 1.08 during the fermentation,(Figure 6) signifying a polymer product with a much narrow molecular weight distribution than the polymer possessing a PDI of 2.0 isolated from *A. eutrophus* by Kawaguchi and Doi (1992).

Example 2: Increasing PHA synthase activity and effect on PHB mw.

Since little or no PHA synthase activity is detected in either the soluble or insoluble fractions of DH5α/pAeT41 lysates, a new construct was designed to test the effect of elevated synthase activity on the molecular weight of PHB. An *E. coli* ribosome binding site was inserted into the plasmid in place of the *A. eutrophus* synthase ribosome binding site to increase synthase activity in the presence of genes encoding β-ketothiolase and acetoacetylCoA reductase. Plasmid pSP2 was constructed containing the strong inducible *trc* promoter, a gene encoding PHA synthase with an optimal *E. coli* ribosome binding site, and genes encoding the thiolase and reductase. A 2.3 kb fragment encoding β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB) was isolated from plasmid pAeT10 (Peoples and Sinskey, 1989b) upon digestion with *Pst* I (Figures 7A-B). The fragment was ligated into the 4.2 kb vector pTRCN (Gerngross et al., 1994) that has been previously digested with *Pst* I and calf intestinal alkaline phosphatase. A plasmid containing *phaA*

adjacent to the *trc* promoter of plasmid pTRCN was isolated and named pSP1A. A 1.7 kb fragment encoding PHA synthase (PhaC) was isolated from plasmid pKAS4 (Gerngross et al., 1994) upon digesting the plasmid with *Eco*R I and *Bam*H I. The fragment was ligated into pSP1A that had been previously digested with *Eco*R I and *Bam*H I. A 8.2 kb plasmid containing *pha*C behind the *trc* promoter, followed by a fragment containing *phaA* and *phaB*, was isolated and named pSP2 (Figure 7B). The presence of a plasmid-borne $lacI^q$ gene on pSP2 allows the transcription of all three PHA biosynthetic genes in this synthetic operon to be tightly controlled by the amount of IPTG added to the culture medium.

Initial batch fermentation of DH5α/pSP2 was performed in the presence of 0.4 mM IPTG which was added to the culture once the absorbance at 600 nm reached 0.6. DH5α/pSP2 induced in this manner consumed all glucose after 18.3 h of fermentation (Figure 1B). Dry cell weight and PHB content were found to be 10.4 and 7.9 g/L at 18.3 and 35.5 h, respectively. A M_w of 2.2 X 10^6 Da was detected after 6.9 h of fermentation and M_w values steadily decreased until 35.5 h at which time isolated polymer possessed a M_w of 1.3 X 10^6, three fold smaller than the 4.0 X 10^6 Da observed at the end of the DH5α/pAeT41 fermentation (Figure 3B). The observed increase in PHB concentration (Figure 1B) during the time span that a decrease in polymer molecular weight was observed (Figure 3B) indicates that the decrease in polymer molecular weight is not due to polymer degradation during the fermentation. Enzyme activities were therefore assayed for the DH5α/pSP2 (0.4 mM IPTG) fermentation to determine if a difference in enzyme activities coincided with the observed differences in M_w of polymer isolated from strains DH5α/pAeT41 and DH5α/pSP2 (0.4 mM IPTG). The most prominent difference in enzyme activity was the presence of detectable synthase activity in DH5α/pSP2 (0.4 mM IPTG), which was predominantly insoluble during the fermentation, yielding a maximum specific activity of 1.4 U/mg in the combined soluble and insoluble

fractions at 13.3 h (Figure 3A). This observation indicated that the concentration of synthase might play a role in determining the size of the polymer produced in the host cell. Soluble synthase in DH5α/pSP2 yielded a maximum activity of 0.11 U/mg at 18.3 h (Figure 2). No reductase activity was detected in the fermentation (Figure 4) and assays for thiolase activity yielded a maximum of 3.6 U/mg at 11.5 h time point of the fermentation (Figure 5). The polymer isolated from the DH5α/pSP2 (0.4 mM IPTG) fermentation possessed a broader molecular weight distribution than polymer isolated from DH5α/pAeT41 as seen from the increase in polydispersity index values which reached a maximum of 1.57 (Figure 6).

To further demonstrate the ability of PHA synthase to regulate the size of PHA produced in a host cell, the transcription of PHA biosynthetic genes in DH5α/pSP2 was elevated by increasing the concentration of the inducing agent, IPTG, to 5 mM. Maximum soluble synthase activity increased from the 0.11 U/mg observed in DH5α/pSP2 induced with 0.4 mM IPTG to 0.21 U/mg when induced with 5 mM IPTG (Figure 2). Likewise, insoluble synthase activity increased, yielding 2.5 U/mg at 16.5 h (Figure 3A). A further reduction of size in the isolated polymer was observed, yielding M_w values of 2.2 X 10^6 Da after 5.8 h of fermentation which decreased to 7.8 X 10^5 Da at 30.2 h, a value five fold smaller than the 4.0 X 10^6 Da observed at the end of the DH5α/pAeT41 fermentation (Figure 3B).

No reductase activity was detected during the fermentation (Figure 4) and thiolase activity yielded a maximum activity of 12.3 U/mg at 16.5 h (Figure 5). Fermentation parameters were comparable to those measured for DH5α/pSP2 induced with 0.4 mM IPTG with dry cell weight and PHB concentration maximum yields of 9.5 and 7.6 g/L, respectively, at 30.2 h (Figure 1C). All glucose was consumed from the fermentation at 16.5 h.

Example 3: Effect of synthase activity on molecular weight distribution.

Analysis of the results in Example 1 by GPC in line with light-scattering showed that the polydispersity index of polymer isolated from DH5α/pSP2 induced with 5 mM IPTG was 2.8 at 25.4 h, indicating a broader molecular weight distribution than polymer samples isolated from DH5α/pAeT41 and DH5α/pSP2 induced with 0.4 mM IPTG (Figure 6A).

The control of molecular weight distribution is reflected by the different polydispersity indices measured for polymer samples isolated from strains DH5α/pAeT41, DH5α/pSP2 induced with 0.4 mM IPTG, and DH5α/pSP2 induced with 5 mM IPTG (Figure 6A). The broadening of distributions upon induction of DH5α/pSP2 with IPTG can be attributed to increasing the concentration of PHA synthase midway through the fermentation. The basal levels of synthase activity before IPTG induction, due to incomplete repression of the trc promoter by the $lacI^q$ gene product, produce a small pool of polymers with high molecular weights. Induction with IPTG midway through the fermentation increases the number of polymer chains competing for substrate producing a new pool of polymers with a shorter chain length. Since the polymer pool now encompasses long chains manufactured before IPTG induction and new shorter chains produced after IPTG induction, the molecular weight distribution of the final polymer product broadens. The extent of synthase induction midway through the fermentation determines the final distribution of the polymer product. This is most evident upon examination of the GPC chromatograms of DH5α/pSP2 induced with 0.4 mM IPTG (Figure 6B) and DH5α/pSP2 induced with 5 mM IPTG (Figure 6C). The chromatograms of both strains reveal the production of a polymer whose median molecular weight exceeds 10^6 Da at an early time point of the fermentation (DH5α/pSP2, 0.4 mM IPTG, 6.9 h; DH5α/pSP2, 5 mM IPTG, 5.8 h). However, upon induction of DH5α/pSP2 with either 0.4 mM or 5 mM IPTG midway through the fermentation, the median molecular weight of polymer isolated from

DH5α/pSP2 induced with 5 mM IPTG (23.4 h) shifts to a lower value than polymer isolated from DH5α/pSP2 induced with 0.4 mM IPTG (18.3 h) yielding a broader molecular weight distribution for the 5 mM sample. Accordingly, varying the extent and time of induction of PHA synthase in a genetic recombinant production strain can be used to produce polymer products with different molecular weight distributions.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Massachusetts Institute of Technology
(ii) TITLE OF INVENTION: Method for Controlling Molecular Weight of Polyhydroxyalkanoates
(iii) NUMBER OF SEQUENCES: 1
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 25 July 1997
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(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: U.S. 08/687,806
(B) FILING DATE: 26-JUL-1996
(C) CLASSIFICATION:
(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: MIT 6867
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(A) TELEPHONE: 404-873-8794
(B) TELEFAX: 404-873-8795
(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4984 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCGCCGCTGC CTCACTCGTC CTTGCCCCTG GCCGCCTGCG CGCGCTCGGC TTCAGCCTTG 240

CGTCGGCGGC GGCCGGGCGT GCCCATGATG TAGAGCACCA CGCCACCGGC GCCATGCCAT 300

ACATCAGGAA GGTGGCAACG CCTGCCACCA CGTTGTGCTC GGTGATCGCC ATCATCAGCG 360

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CAGGGCAGCG GCGCAGCCAG CACCATGTTC GTGCAGCGCG GCCCTCGCGG GGGCGAGGCT 4980

GCAG 4984

We claim:

1. A method for controlling molecular weight and dispersity of polyhydroxyalkanoates comprising

controlling the amount of polyhydroxyalkanoate synthase activity in a host expressing the synthase and providing substrate for polymerization of polyhydroxyalkanoates, wherein the molecular weight of the polyhydroxyalkanoate is inversely proportional to the activity of the polyhydroxyalkanoate synthase activity.

2. The method of claim 1 wherein the amount of synthase activity is controlled by engineering the gene encoding the synthase to be under the control of an inducible promoter and regulating the expression of the synthase by inducing the promoter.

3. The method of claim 1 wherein the activity of the synthase is altered over time to increase the molecular weight dispersity of the polyhydroxyalkanoate.

4. The method of claim 1 wherein the synthase activity is maintained at substantially the same specific activity over a period of time to yield a polyhydroxyalkanoate having a molecular weight dispersity of between 1.02 and 2.81.

5. The method of claim 1 wherein the polyhydroxyalkanoate has a molecular weight range of between 700,000 and 4,000,000 g/mol.

6. The method of claim 1 wherein the amount of synthase activity is controlled by increasing the activity of the synthase.

7. An expression system for the synthesis of polyhydroxyalkanoate comprising a synthase under the control of an inducible promoter.

8. The expression system of claim 7 comprising a host selected from the group consisting of plants and bacteria expressing a reductase and thiolase.

9. A polyhydroxyalkanoate composition having a molecular weight dispersity of between 1.02 and 2.81.

10. The polyhydroxyalkanoate composition of claim 9 wherein the polyhydroxyalkanoate has a molecular weight range of between 700,000 and 4,000,000 g/mol.

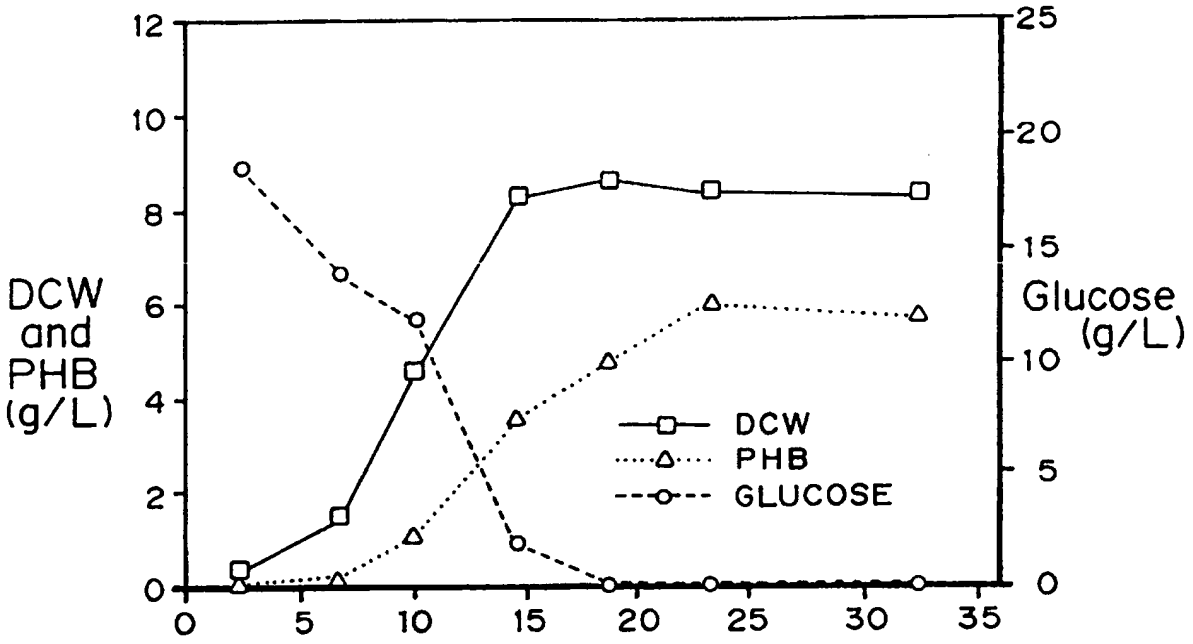

DCW and PHB (g/L)
Glucose (g/L)
DCW
PHB
GLUCOSE
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
0
5
10
15
20
25

FIG. 1a

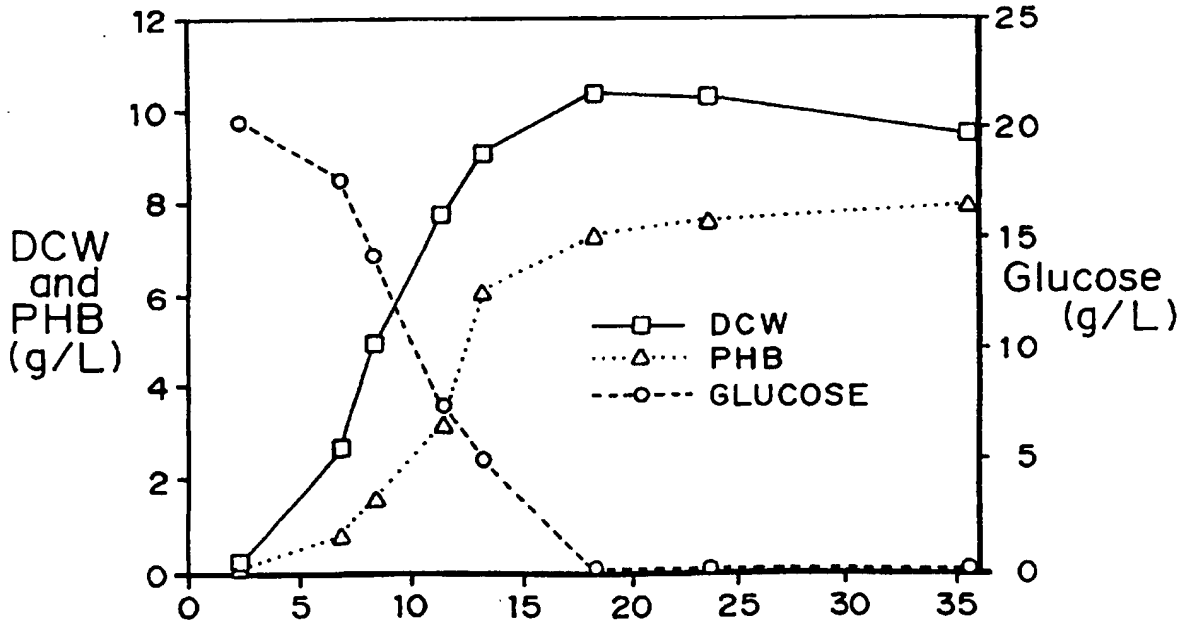

DCW and PHB (g/L)
Glucose (g/L)
DCW
PHB
GLUCOSE
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
0
5
10
15
20
25

FIG. 1b

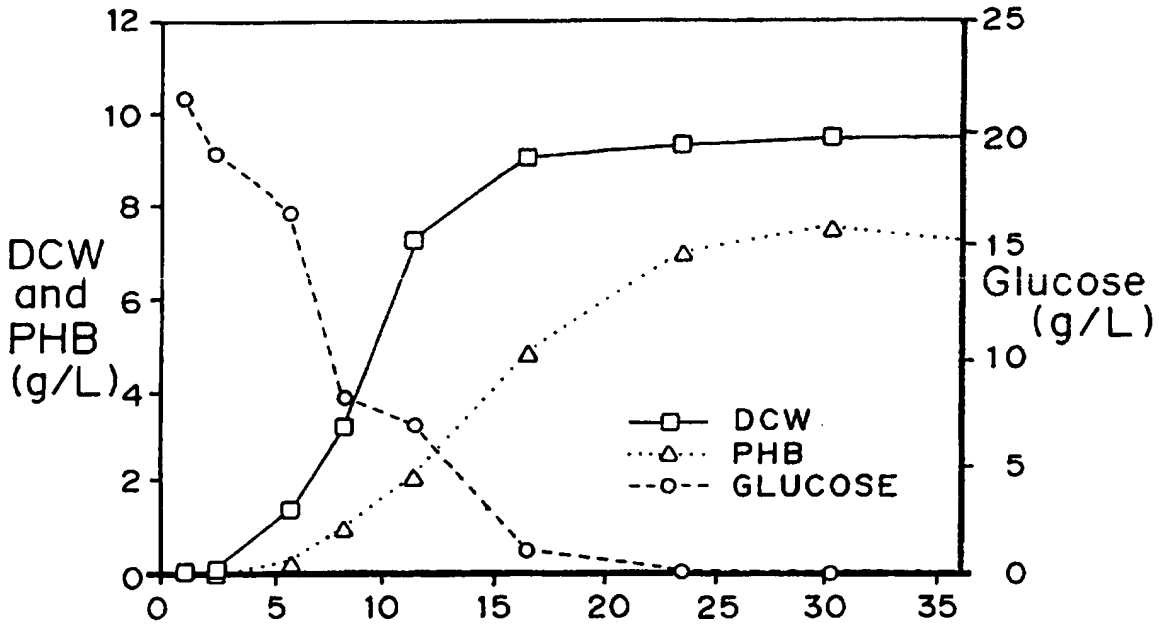

DCW and PHB (g/L)
Glucose (g/L)
DCW
PHB
GLUCOSE
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
0
5
10
15
20
25

FIG. 1c

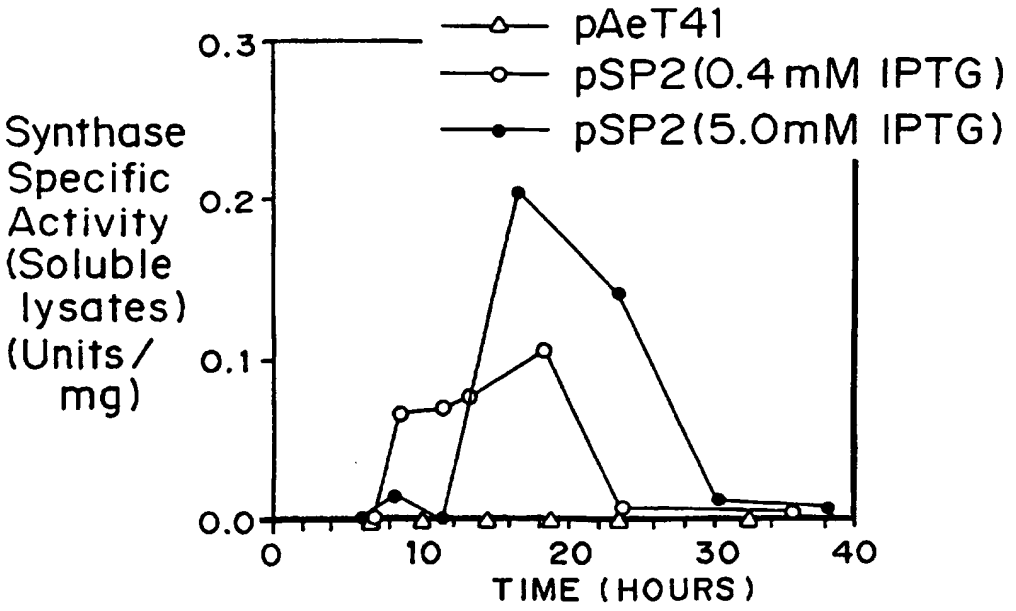

pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0mM IPTG)
Synthase Specific Activity (Soluble lysates) (Units/ mg)
0.0
0.1
0.2
0.3
0
10
20
30
40
TIME (HOURS)

FIG. 2

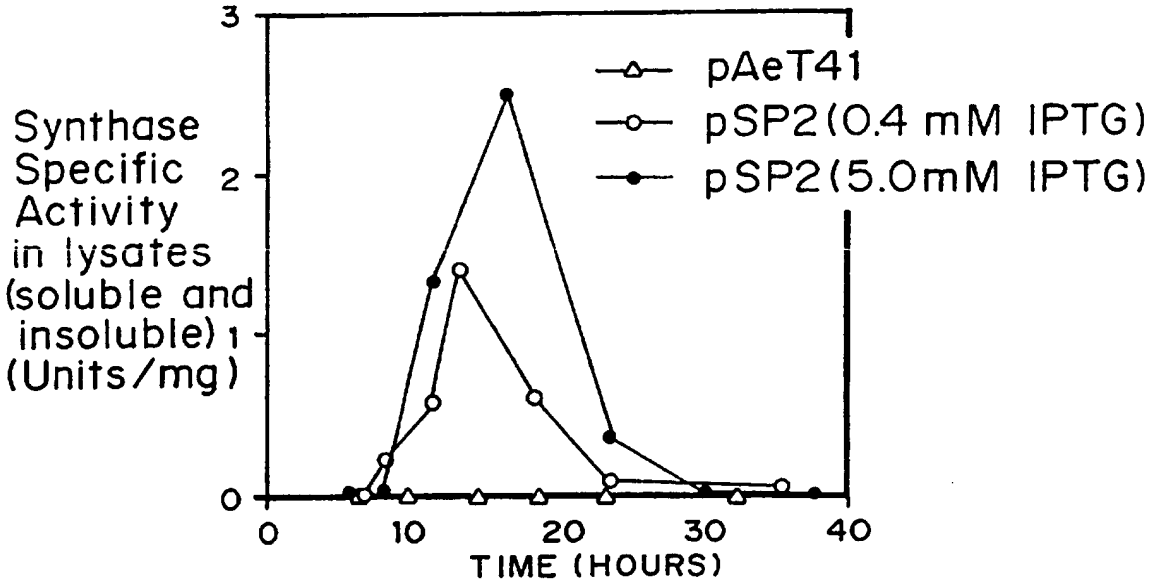

Synthase Specific Activity in lysates (soluble and insoluble) (Units/mg)
3
2
1
0
0
10
20
30
40
TIME (HOURS)
pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0mM IPTG)

FIG. 3a

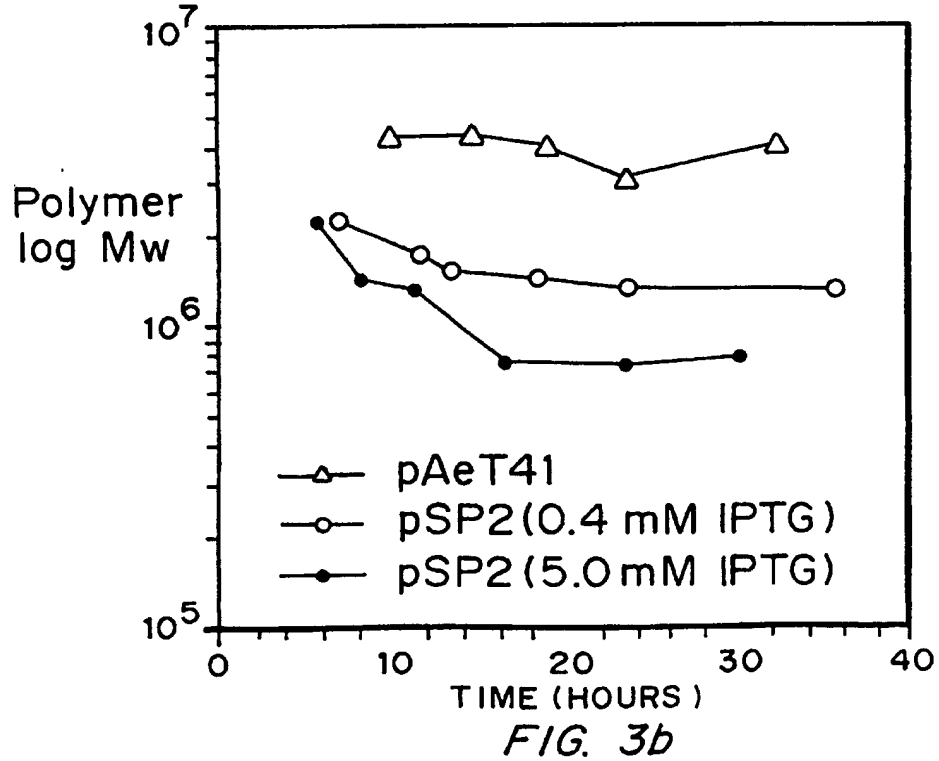

Polymer log Mw
10^7
10^6
10^5
0
10
20
30
40
TIME (HOURS)
pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0mM IPTG)

FIG. 3b

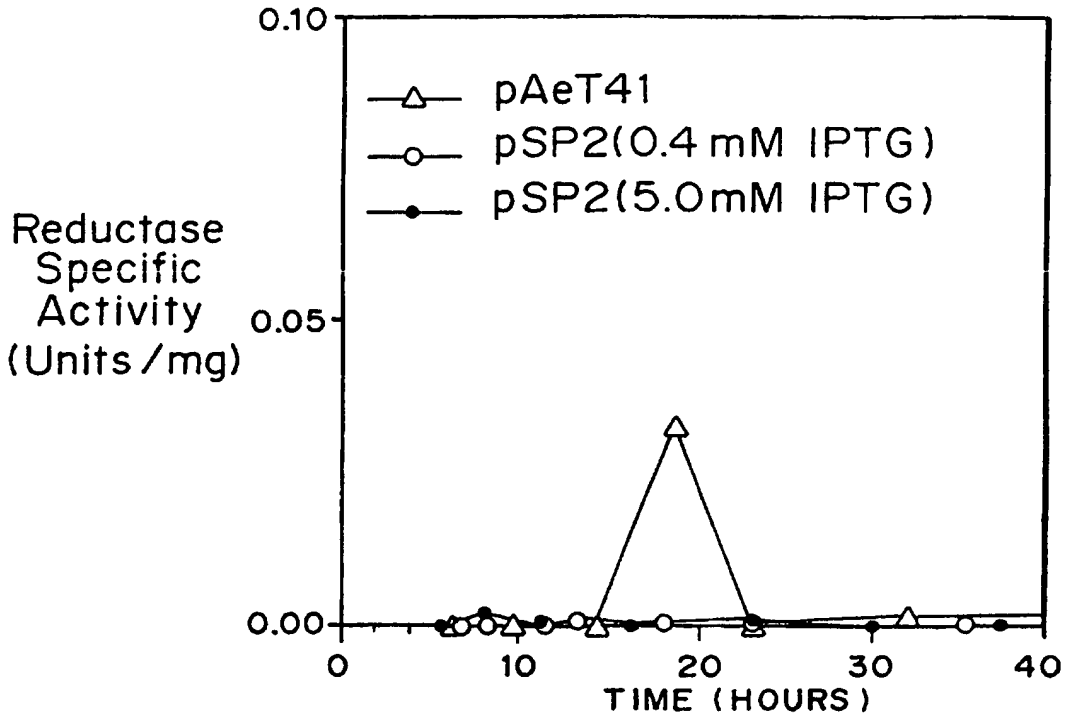

0.10
0.05
0.00
Reductase Specific Activity (Units/mg)
pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0mM IPTG)
0
10
20
30
40
TIME (HOURS)

FIG. 4

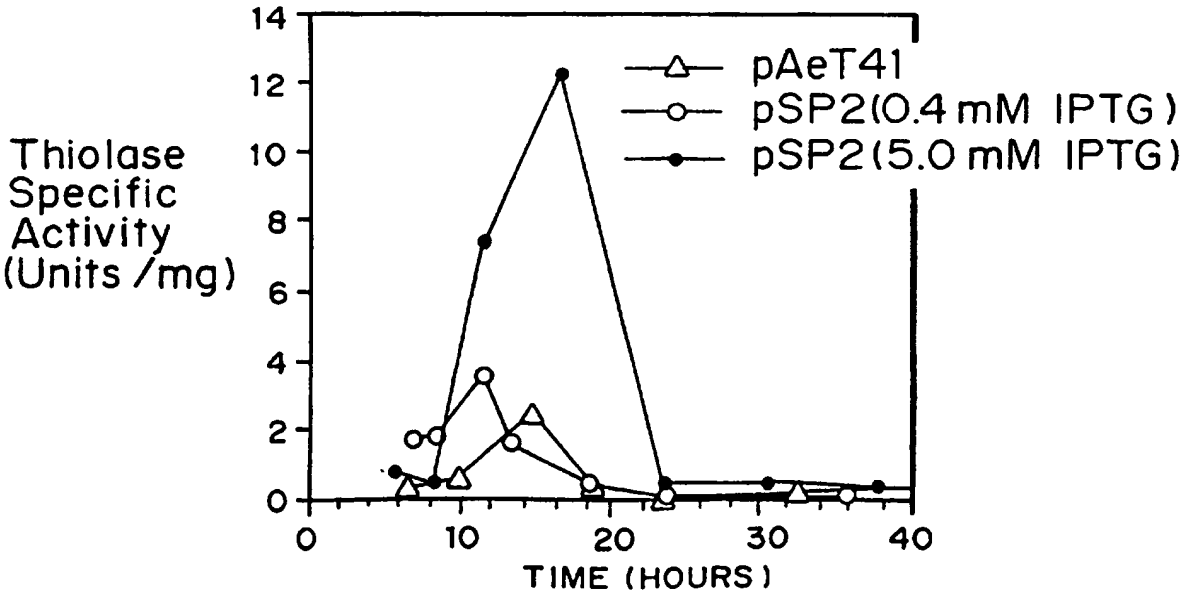

14
12
10
8
6
4
2
0
Thiolase Specific Activity (Units/mg)
pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0 mM IPTG)
0
10
20
30
40
TIME (HOURS)

FIG. 5

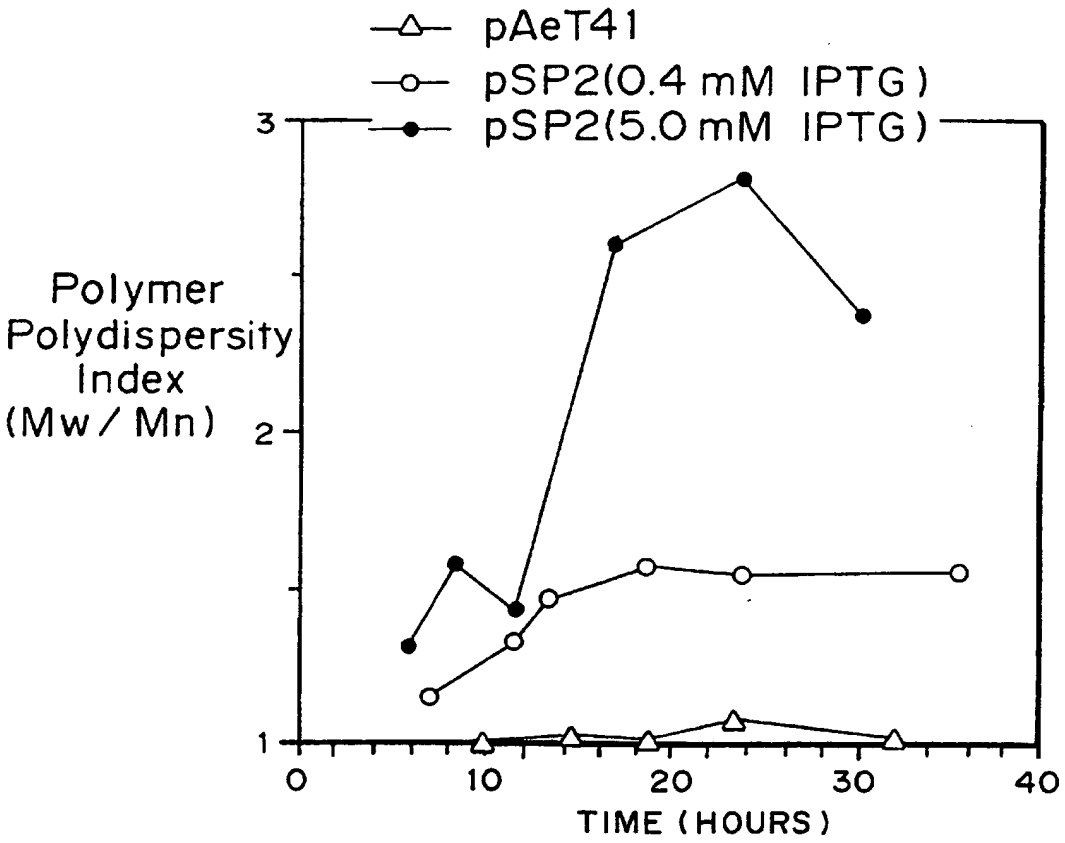

pAeT41
pSP2(0.4 mM IPTG)
pSP2(5.0 mM IPTG)
Polymer Polydispersity Index (Mw / Mn)
3
2
1
0
10
20
30
40
TIME (HOURS)

FIG. 6a

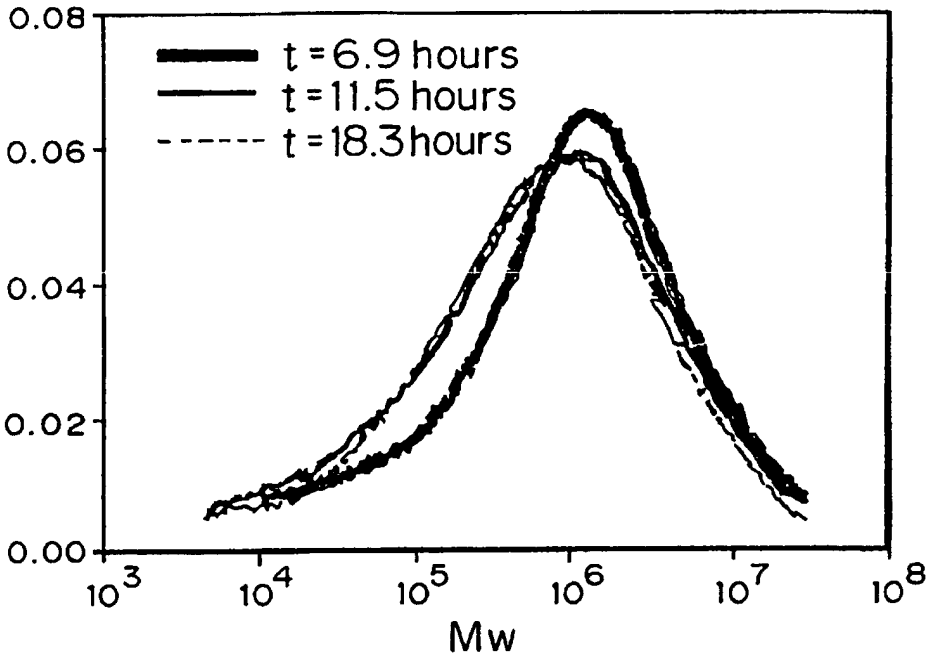

0.08
0.06
0.04
0.02
0.00
t = 6.9 hours
t = 11.5 hours
t = 18.3 hours
10^3
10^4
10^5
10^6
10^7
10^8
Mw

FIG. 6b

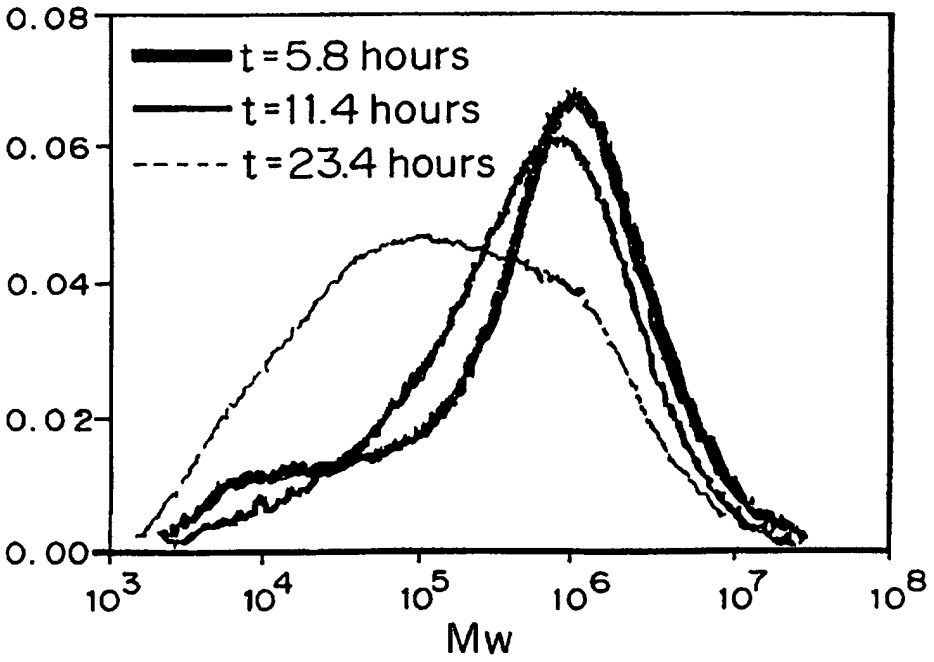

0.08
0.06
0.04
0.02
0.00
t = 5.8 hours
t = 11.4 hours
t = 23.4 hours
10^3
10^4
10^5
10^6
10^7
10^8
Mw

FIG. 6c

FIG. 7a

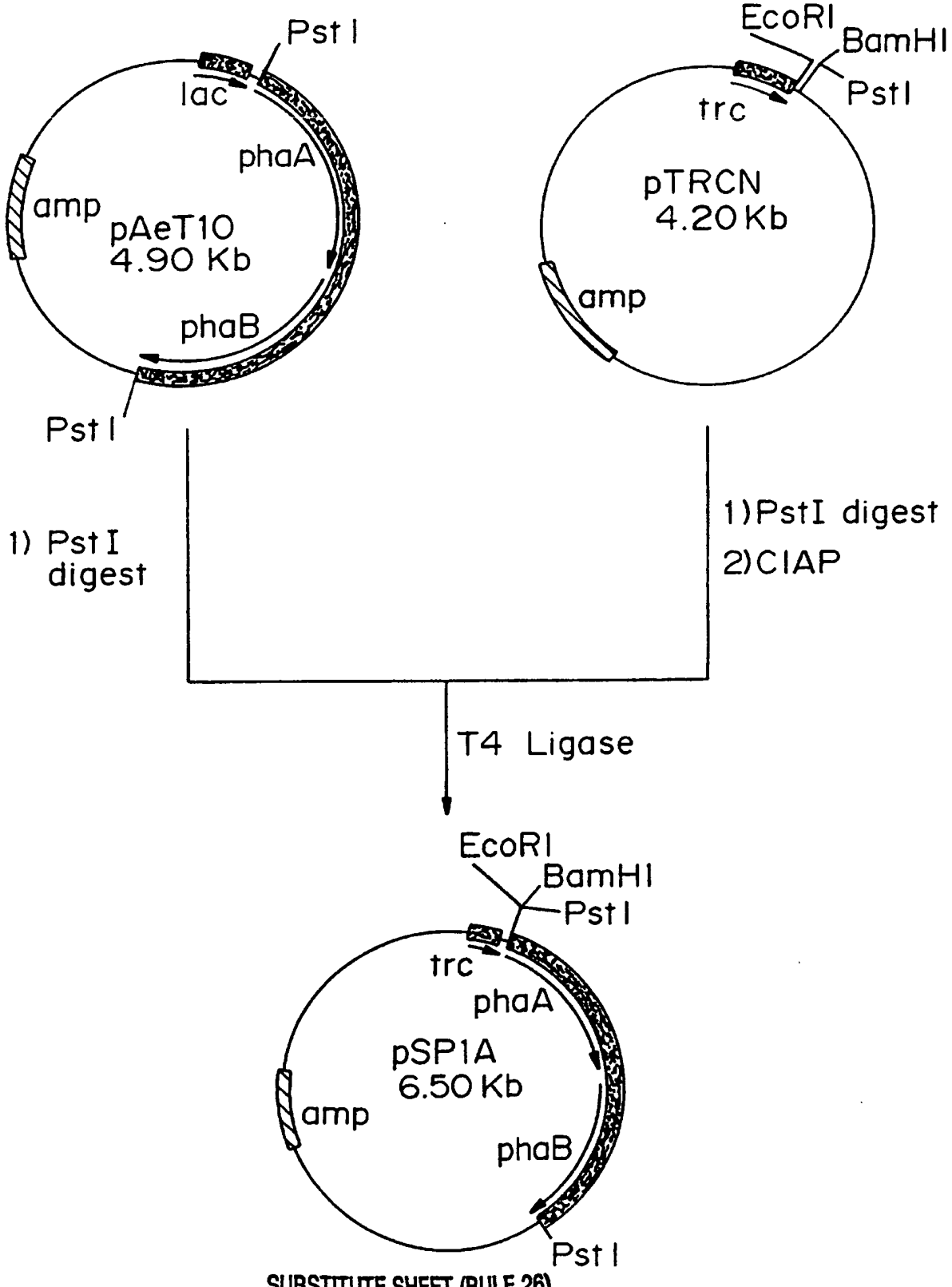
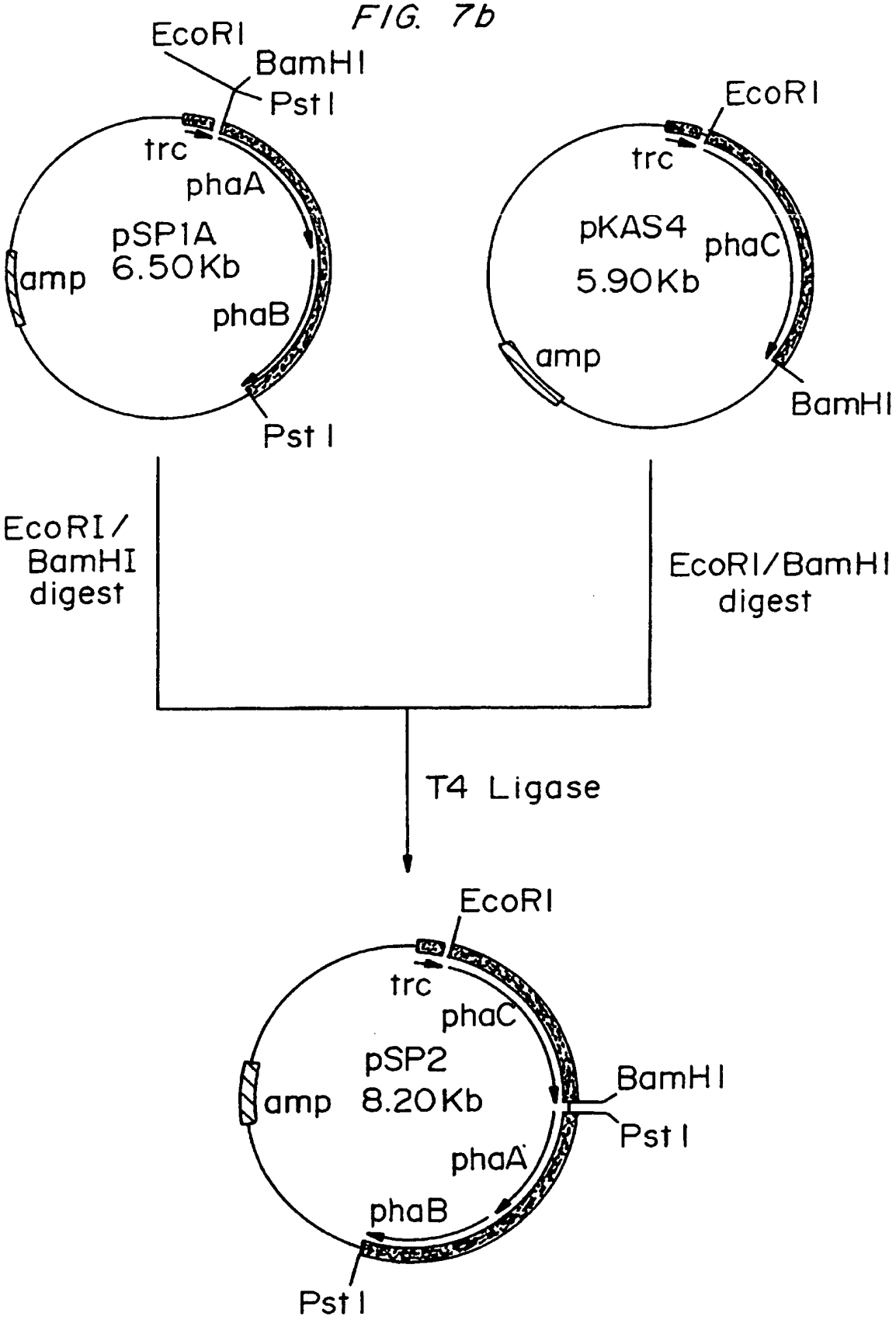

Pst I
lac
phaA
amp
pAeT10
4.90 Kb
phaB
Pst I
EcoRI
BamHI
PstI
trc
pTRCN
4.20Kb
amp
1) Pst I
digest
1)PstI digest
2)CIAP
T4 Ligase
EcoRI
BamHI
PstI
trc
phaA
pSP1A
6.50 Kb
amp
phaB
Pst I

FIG. 7b


EcoRI
BamHI
PstI
trc
phaA
pSP1A
6.50Kb
amp
phaB
PstI
EcoRI
trc
pKAS4
phaC
5.90Kb
amp
BamHI
EcoRI/
BamHI
digest
EcoRI/BamHI
digest
T4 Ligase
EcoRI
trc
phaC
pSP2
8.20Kb
amp
BamHI
PstI
phaA
phaB
PstI

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 97/13301

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12P7/62 C12N9/00 //C12N1/21,C12N5/14

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 21257 A (INNOVATIVE TECH CENTER) 10 August 1995 see page 11; claims	1
A	WO 95 21260 A (INNOVATIVE TECH CENTER) 10 August 1995 see page 11; claims	1
A	WO 96 05316 A (MASSACHUSETTS INST TECHNOLOGY ;METABOLIX INC (US)) 22 February 1996 see claims	1
A	WO 91 00917 A (MASSACHUSETTS INST TECHNOLOGY) 24 January 1991 see claims	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
1 December 1997	19/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Delanghe, L

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INTERNATIONAL SEARCH REPORT

Internat ' Application No
PCT/US 97/13301

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	CHEMICAL ABSTRACTS, vol. 124, no. 5, 29 January 1996 Columbus, Ohio, US; abstract no. 50315, KOIZUMI, FUMITAKE ET AL: "Molecular weight of poly(3-hydroxybutyrate) during biological polymerization in Alcaligenes eutrophus" XP002048625 see abstract & PLAST. ENG. (N. Y.) (1995), 29(DEGRADABLE POLYMERS, RECYCLING, AND PLASTICS WASTE MANAGEMENT), 167-82 CODEN: PLENEZ;ISSN: 1040-2527,	1
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